Summer 2007

Expression and characterization of the Na-K-Cl cotransporter, masBSC, in Sf9 cells

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ABSTRACT

Ion transport across cell membranes plays an important role in regulating cell volume and intracellular ion levels as well as in transepithelial ion secretion and absorption. The Na-K-Cl cotransporter, masBSC, found in the tobacco hornworm caterpillar, *Manduca sexta*, is suspected to function similarly to the well-studied mammalian Na-K-Cl cotransporters, NKCC1 and NKCC2. In order to characterize masBSC, this protein was expressed in Sf9 cells (cells derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda)* by transfecting cultures with a vector construct containing the masBSC gene (cloned from the Malpighian tubules of *M. sexta*). Rubidium influx assays were then performed in order to measure ion transport by these cells. Results suggest that masBSC cultures transport more ions compared to control cultures (cells transfected with the pIB vector alone) and therefore that exogenous expression of masBSC was successful. Additionally, to gain insight as to how growing organisms cope with decreasing surface area to volume ratios, endogenous expression of masBSC in *M. sexta* was measured via real-time PCR. Results show that masBSC expression is higher in the foregut of 4th instar larva.

INTRODUCTION

- Na-K-Cl cotransport is the coupled movement of Na^+, K^+, and Cl^- ions across cell membranes and has been found in many insect epithelia, including that of *Manduca sexta*, the tobacco hornworm caterpillar (Bowles and Gillen, 2001).
- This cotransport plays an important role in regulating cell volume and intracellular ion levels as well as in transepithelial ion secretion and absorption. A transport protein identified in *Manduca sexta*, masBSC, has been hypothesized to function as a Na-K-Cl cotransporter (Reagan, 1995).
- Mammalian Na-K-Cl cotransporters (NKCC1 and NKCC2) have been studied in depth, and their structure, function, and ion specificities are known. Both proteins appear to consist of a hydrophobic region, consisting of 12 α-helices, that is "flanked by an amino- and a carboxy-terminal region" (Russell, 2000).
- Previous studies have shown that masBSC is widely found in *Manduca sexta* tissues, specifically along the apical wall of midgut epithelial cells (Gillen *et al*., 2006).
- Given that masBSC and the mammalian transporters share significant sequence specificity, a reasonable hypothesis is that masBSC functions as a Na-K-Cl cotransporter in much the same way as the mammalian cotransporters.
- Characterization of masBSC via cation chromatography, in which we can measure the concentration of specific ions present in particular cells, will enable us to understand exactly how masBSC works and which ions are actually involved in the transport processes of this protein.

METHODS

Expression in Sf9 cells**:**
The masBSC expression vector made by Gillen *et al*. (2006) as well as the pIB/V5-HIS expression vector alone were transfected into Sf9 cultures using Cellfectin (Invitrogen). The transfected cells were selected for based on blasticidin resistance and continued to grow in TNM-FH complete media containing blasticidin.

Functional Experiment – Ion characterization:
masBSC and pIB Sf9 cultures were grown up on 24-well plates. After removing the TNM-FH media, all wells were first treated with a rinse solution. The cultures were then treated with flux solutions varying in both rubidium concentration and in exposure time. Following the flux, cultures received rinses of 100 mM $MgCl_2$. Intracellular ions levels were collected by freezing the 24-well plate and treating with absolute ethanol. Ion samples (diluted in Milli-Q water) were then loaded into the Dionex chromatography system and subsequently measured.

Endogenous masBSC expression:
Tissues from three regions of the midgut (anterior, middle, and posterior) as well as from the foregut, hindgut, and Malpighian tubules were collected from 4th and 5th instar *M. sexta* larva. Total RNA was extracted using RNA STAT-60 (Tel-test, Inc.). The RNA was rid of DNA contamination using Turbo DNA-*free* (Ambion) and converted to cDNA using the TaqMan RT-PCR kit (Applied Biosystems). Real-time PCR was then performed using these cDNAs, *M. sexta* 18S and masBSC primers, the SYBR green real-time PCR kit (Applied Biosystems), and the ABI 7500 instrument (Applied Biosystems).

RESULTS

Expression and Characterization Data:

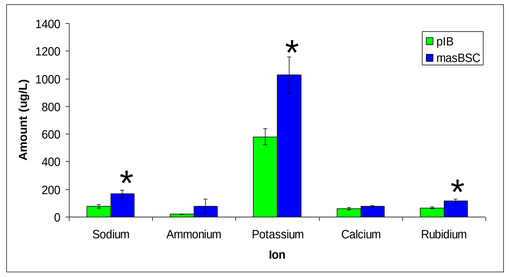

Figure 1. Results of a rubidium influx assay. Sf9 cells transfected with the masBSC vector construct or the pIB vector alone were treated with 5mM Rb^+ for 5 minutes. Intracellular ion levels were measured using the Dionex chromatography system. Mean ± SE, n = 6. Significant differences indicated by stars (t-test, $p < 0.05$).

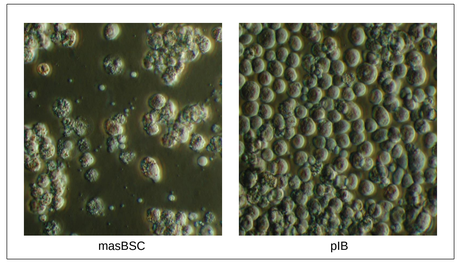

Figure 2. Micrographs of Sf9 cultures transfected with either the pIB vector alone or the masBSC vector construct. Magnification = 400x.

Real-time PCR Data:

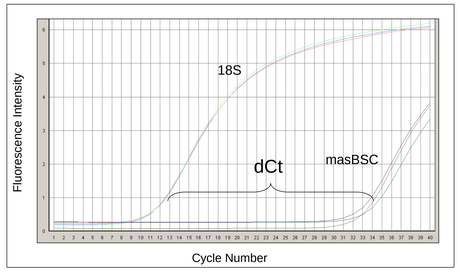

Figure 3. Real-time PCR amplification plot. Amplification of 4th instar midgut cDNAs by masBSC1 and mas18S primers. Amplification of the cDNAs was performed in triplicate. A larger dCt indicates a lower mRNA level.

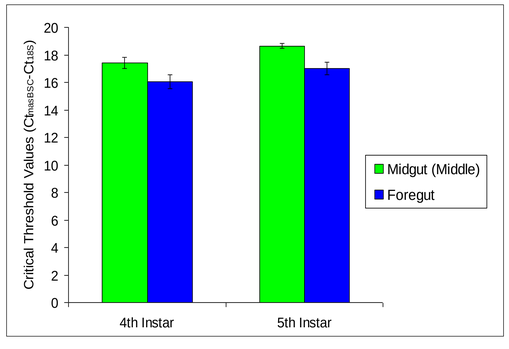

Figure 4. Critical threshold values (dCt) for amplification of *Manduca sexta* cDNAs (from midgut and foregut tissues of 4th and 5th instar caterpillars) with masBSC1 primers. Mean ± SE, n = 4 except for 5th instar midgut, in which n = 3. Significant differences between instars and tissues (2-way ANOVA, $p < 0.05$).

DISCUSSION

masBSC Expression and Characterization:
- Data suggest the successful transfection and exogenous expression of the Na-K-Cl cotransporter, masBSC.
- Intracellular ion content in Sf9 cells transfected with the masBSC vector were higher than in those cells transfected with the pIB vector alone (control). This suggests that masBSC Sf9 cells were actively expressing the masBSC cotransporter and were therefore able to transport more ions into the cell than cells lacking the cotransporter.
- Cells transfected with the masBSC vector showed signs of slowed growth. This may suggest that expression of the masBSC cotransporter in Sf9 cells decreases the viability of the cells and reduces their ability to grow and develop.
- Future investigation into the stable expression of this cotransporter may be directed toward finding a different cell type that better supports exogenous expression of this protein or toward producing a stable clonal cell line.

Real-time PCR:
- Endogenous expression of masBSC varies between tissue type and instar in *M. sexta*.
- Measuring the expression of this cotransporter in different tissues and during different growth stages may help to investigate how growing organisms cope with a decreased surface area to volume ratio.
- Expression of masBSC was higher in the foregut than in the midgut and higher in 4th instar larva than in 5th instar larva. Though cotransporter expression was hypothesized to increase across instars, previous work has found a high expression of masBSC in the foregut (Gillen *et al*., 2006).
- More real-time PCR should be performed with masBSC in order to verify these results. Work should also be done to measure the expression levels of other transporters found in the gut of *M. sexta* so that transporter expression can be compared.

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ACKNOWLEDGEMENTS

I would like to thank Professor Gillen for his support and guidance throughout this project, as well as Lexi White and Michael Northcutt. The Kenyon College Summer Science Program and the Kenyon College Department of Biology provided funding for this research.